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Impaired Bone Resorption to Prostaglandin E_2 in Prostaglandin E Receptor EP4-knockout Mice*

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Prostaglandin E_2 (PGE_2) acts as a potent stimulator of bone resorption. In this study, we first clarified in normal ddy mice the involvement of protein kinase A and induction of matrix metalloproteinases (MMPs) in PGE_2-induced bone resorption, and then identified PGE receptor subtype(s) mediating this PGE_2 action using mice lacking each subtype (EP1, EP2, EP3, and EP4) of PGE receptor. In calvarial culture obtained from normal ddy mice, both PGE_2 and dibutyryl cyclic AMP (Bt_2cAMP) stimulated bone resorption and induced MMPs including MMP-2 and MMP-13. Addition of an inhibitor of protein kinase A, H89, or an inhibitor of MMPs, BB94, significantly suppressed bone-resorbing activity induced by PGE_2. In calvarial culture from EP1-, EP2-, and EP3-knockout mice, PGE_2 stimulated bone resorption to an extent similar to that found in calvaria from the wild-type mice. On the other hand, a marked reduction in bone resorption to PGE_2 was found in the calvarial culture from EP4-knockout mice. The impaired bone resorption to PGE_2 was also detected in long bone cultures from EP4-knockout mice. Bt_2cAMP greatly stimulated bone resorption similarly in both wild-type and EP4-knockout mice. Induction of MMP-2 and MMP-13 by PGE_2 was greatly impaired in calvarial culture from EP4-knockout mice, but Bt_2cAMP stimulated MMPs induction similarly in the wild-type and EP4-knockout mice. These findings suggest that PGE_2 stimulates bone resorption by a cAMP-dependent mechanism via the EP4 receptor.

Prostaglandins (PGs)[1] are produced in the bone mainly by osteoblasts and stimulate both bone formation and resorption (1–3). Among several PGs produced, PGE_2 is a major product, and its production by osteoblasts is regulated by several cytokines including interleukin-1 (IL-1). We previously reported that cytosolic phospholipase A_2 is responsible for the release of arachidonic acid in osteoblasts, and that the conversion of arachidonic acid to PGE_2 is then catalyzed by cyclooxygenase (COX)-2 induced in response to IL-1 (4). *In vitro*, PGE_2 production primarily leads to bone resorption (3, 5); PGE_2 stimulates adenylate cyclase in osteoblasts, induces osteoclast formation in co-cultures of bone marrow cells and osteoblasts, and stimulates bone resorption in calvarial cultures (5, 6). In contrast, PGE_2 stimulates both bone formation and resorption *in vivo*. The mechanism of the anabolic effect of PGE_2 still remains unclarified.

The actions of PGE_2 are mediated by rhodopsin-type receptors specific to this PG. There are four subtypes of PGE receptors designated as EP1, EP2, EP3, and EP4 that are encoded by different genes and expressed differently in each tissue (7–10). The intracellular signaling also differs among the receptor subtypes; EP1 is coupled to Ca^{2+} mobilization, and EP3 inhibits adenylate cyclase, whereas both EP2 and EP4 stimulate adenylate cyclase (7, 8). To identify the physiological functions of each EP receptor subtype, we have generated mice lacking respective receptors by homologous recombination (11–13). Loss of EP4 is not lethal *in utero*, but most EP4 (−/−) neonates die within 72 h after birth due to patent ductus arteriosus, suggesting that the EP4 receptor plays a role in the regulation of the patency of this vessel (11, 14). On the other hand, EP3 (−/−) mice failed to show a febrile response to various pyrogens, suggesting that PGE_2 mediates fever generation by acting on the EP3 receptor (12).

We previously reported that cytokines with bone-resorbing activity such as IL-1 and IL-6 greatly induce the expression of matrix metalloproteinases (MMPs) including MMP-13 (type 3 collagenase) and MMP-2 (gelatinase A) in mouse calvarial cultures, and that the potency of various cytokines to induce MMPs is closely correlated to their bone-resorbing activity that involves the degradation of bone matrix (15). Using collagenase-resistant mutant mice, Zhao *et al.* (16) recently reported that the cleavage of type I collagen by collagenase such as MMP-13 is essential for the induction of osteoclastic bone resorption. Therefore, monitoring the induction of MMPs in mouse calvarial cultures appears to be a suitable measure for the bone-resorbing activity.

In this study, we used EP1-, EP2-, EP3-, and EP4-knockout mice and examined the mechanism of action of PGE_2 in bone resorption. We identified the EP4 subtype of PGE receptor responsible for transducing signals for bone-resorbing activity of PGE_2.

MATERIALS AND METHODS

Animals and Reagents—Newborn mice of the ddy strain were obtained from Japan SLC Inc. (Shizuoka, Japan). Mice lacking EP1, EP2, EP3, and EP4 were generated, and homozygote, heterozygote and wild-type mice of the F2 progeny were used (11–13). To examine the genotype of each mouse, polymerase chain reaction analysis was performed

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[1] The abbreviations used are: PG, prostaglandin; Bt_2cAMP, dibutyryl cAMP; MMP, matrix metalloproteinase; PAGE, polyacrylamide gel electrophoresis; IL, interleukin; TRAP, tartrate-resistant acid phosphatase; FITC, fluorescein isothiocyanate; COX, cyclooxygenase.

on DNA extracted from the tail or brain of neonates, using the oligonucleotide primers designed to detect the respective EP locus and Neo cassette, as reported previously (11–13). PGE_2 and dibutyryl cyclic AMP (Bt_2cAMP) were obtained from Sigma. H89, an inhibitor of protein kinase A, was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). BB94, an inhibitor of MMPs, was kindly donated by British Biotech Pharmaceuticals Ltd. (Oxford, UK). All other chemicals were of analytical grade.

Bone Resorption Assay—One-day-old mice were killed, and their calvariae, radii, and ulnae were aseptically isolated and dissected free of suture tissues. For calvarial culture, the calvaria were divided into halves and cultured for 24 h at 37° C under 5% CO_2 in air in 0.12 ml of BGJb medium (Life Technologies, Inc.) containing 1 mg/ml bovine serum albumin (fraction V, Sigma). After pre-culture for 24 h, each half calvaria was transferred to fresh medium with or without indicated reagents, and cultured for an additional 72 h. For long bone culture, radii and ulna were cultured in the same condition. The bone-resorbing activity was determined by measuring the concentration of calcium in the conditioned medium using a calcium kit (Calcium C test; Wako Pure Chemicals, Osaka, Japan). The activity was expressed as an increase in medium calcium (15). To detect osteoclasts, calvaria were fixed with 10% formalin and stained for tartrate-resistant acid phosphatase (TRAP). The bone-resorbing activity expressed as an increase in medium calcium changed in parallel with the number of TRAP-positive osteoclasts in cultured calvaria (5, 15).

Northern Blot Analysis—Total RNA was extracted from mouse calvariae using the acid guanidium-phenol-chloroform method (15). For Northern blotting, 20 μg of total RNA was resolved by electrophoresis on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane, which was then hybridized with a ^{32}P-labeled cDNA probe as reported (4, 15). A 485-base pair fragment of mouse MMP-13 cDNA and a 250-base pair fragment of human MMP-2 cDNA were used as probes (15).

Gelatin Zymography—Gelatinase activity in the conditioned medium of calvarial cultures was analyzed by zymography as reported previously (15). Aliquots (10 μl) were mixed with 5 μl of non-reducing SDS-PAGE sample buffer, then subjected to SDS-PAGE using 10% polyacrylamide gel containing 0.6 mg/ml gelatin. After electrophoresis, gels were incubated for 1 h in washing buffer (50 mM Tris-HCl containing 5 mM $CaCl_2$ 1 mM $ZnCl_2$, and 2.5% Triton X-100) to remove SDS, and then in the same buffer without Triton X-100 for 3 h. Gels were then stained with Coomassie Brilliant Blue to detect enzyme activity as a clear zone in a dark stained background.

Western Blot Analysis—An aliquot of the conditioned medium of calvarial cultures was subjected to SDS-PAGE using 10% polyacrylamide gels, and separated proteins were transferred to a polyvinylidene difluoride membrane (Hybond-PNDF, Amersham Pharmacia Biotech). The membrane was first incubated for 18 h with 5% skim milk in phosphate-buffered saline containing 0.1% Tween 20 at 4° C to block nonspecific binding, and then incubated for 2 h with polyclonal rabbit anti-MMP-13 antibody (kindly donated by Dr. Gillian Murphy). After incubation with horseradish peroxidase-conjugated donkey anti-rabbit Ig G for 1 h, immunoreactive bands were stained by an ECL system (Amersham Pharmacia Biotech).

Assay of the Collagenase and Gelatinase Activities—To measure the collagenase and gelatinase activities, conditioned media of calvarial cultures were treated for 4 h with 4-aminophenylmercuric acetate, which activates pro-MMPs to their respective active forms. The collagenase and gelatinase activities were determined by measuring the degradation of fluorescent isothiocyanate (FITC)-labeled type I and type IV collagen using a type I or type IV collagenase assay kit (Yagai Co.). One unit of these activities degrades 1 μg of each collagen/min at 37° C.

Statistical Analysis—Statistical analysis was carried out by Student's *t* test, and the data are expressed as means ± S.E.

RESULTS AND DISCUSSION

PGE_2 markedly stimulates osteoclast-mediated bone resorption *in vitro* by enhancing both the formation and function of osteoclasts. We previously showed in mouse bone marrow cultures that PGE_2 promoted osteoclast formation by a cyclic AMP (cAMP)-mediated mechanism (2, 5). In fact, PGE_2 acts on osteoblasts to elicit cAMP production. We also found previously that IL-1 markedly induced the expression of MMPs including MMP-13, MMP-2, and MMP-3 (stromelysin), and that this induction was associated with an increase in bone-resorbing activity in mouse calvarial cultures (15). In this study, we first used cultures of calvaria isolated from normal ddy mice and examined the involvement of protein kinase A and MMP induction in PGE_2-induced bone resorption. Consistent with the previous findings (2, 5), both PGE_2 and Bt_2cAMP stimulated bone resorption in a concentration-dependent manner in mouse calvarial cultures (Fig. 1*A*). Addition of an inhibitor of protein kinase A, H89, markedly suppressed PGE_2-induced bone resorption (Fig. 1*B*), indicating that a cAMP-dependent mechanism is essential for bone resorption by PGE_2 in the organ culture system. The bone-resorbing activity in the control culture without PGE_2 was not suppressed by H89 at all (Fig. 1*B*). To examine the involvement of the induction of MMPs in PGE_2-induced bone resorption in this culture system, we subjected cultured calvaria and its conditioned medium to Northern blot analysis, Western blot analysis, and gelatin zymography of MMPs. Both PGE_2 and Bt_2cAMP markedly increased expression of both MMP-13 and MMP-2 mRNA on day 2 in mouse calvarial cultures (Fig. 1, *C* and *D*). Consistently, Western blot analysis showed the accumulation of MMP-13 protein in the medium of calvariae treated with PGE_2 or Bt_2cAMP (Fig. 1*C*), and gelatin zymography revealed that MMP-2 activity that was detected only marginally in the control culture was greatly enhanced by treatment with either PGE_2 or Bt_2cAMP (Fig. 1*D*). The potency of PGE_2 and Bt_2cAMP in induction of MMPs was very similar to that of IL-1 (15). To confirm that the MMPs expressed in mouse calvariae had functional enzymatic activities, the collagenase and gelatinase activities in the conditioned media were determined by measuring the degradation of FITC-labeled type I and type IV collagen. PGE_2 and Bt_2cAMP markedly increased both collagenase and gelatinase activities (Fig. 1, *C* and *D*). To evaluate the role of MMP induction in PGE_2-induced bone resorption, we added BB94, an inhibitor of MMPs, to mouse calvarial cultures treated with PGE_2, and examined its effects. As shown in Fig. 1*B*, BB94 markedly suppressed bone-resorbing activity induced by PGE_2, but the activity of the control culture without PGE_2 was not altered by the inhibitor. These results indicate that the expression of MMPs is essential for bone resorption, likely by promoting the degradation of bone matrix, and that monitoring the induction of MMPs is a useful measure for the bone-resorbing activity in mouse calvarial cultures.

PGE_2 thus causes bone resorption of cultured calvaria, and protein kinase A and MMPs induction are involved in the process. However, the PGE receptor subtype(s) mediating this action remains unknown. To identify the responsible receptor subtype(s), we isolated calvariae from mice deficient individually in EP1, EP2, EP3, and EP4 receptor, and subjected them to bone resorption to PGE_2. In calvariae from EP1(−/−), EP2(−/−), and EP3(−/−) mice, PGE_2 stimulated bone resorption as in wild-type mice. In contrast, a marked reduction in bone resorption was found in calvarial culture from EP4 (−/−) mice (Fig. 2*A*). The dose-dependent induction of bone-resorbing activity by 0.1–10 μM PGE_2 was greatly diminished in EP4(−/−) mice (Fig. 2*B*). PGE_2 induced bone resorption in calvaria from heterozygote EP4 (+/−) mice to the same level as that found in the bone from wild-type mice (data not shown). To exclude a possibility of other defect(s) in the signal transduction pathway, we examined the bone-resorbing activity of Bt_2cAMP in calvarial cultures from EP4 (−/−) and wild-type mice. As shown in Fig. 2*C*, Bt_2cAMP stimulated bone resorption similarly in wild-type and EP4 (−/−) mice. Thus, the downstream pathway from cAMP to the bone resorption appeared intact in EP4 (−/−) mice, and the reduction of PGE_2-induced bone resorption in these mice is likely due to a lack of EP4 receptor (Fig. 2, *B* and *C*). Calvaria from EP4(−/−) and wild-type mice were cultured

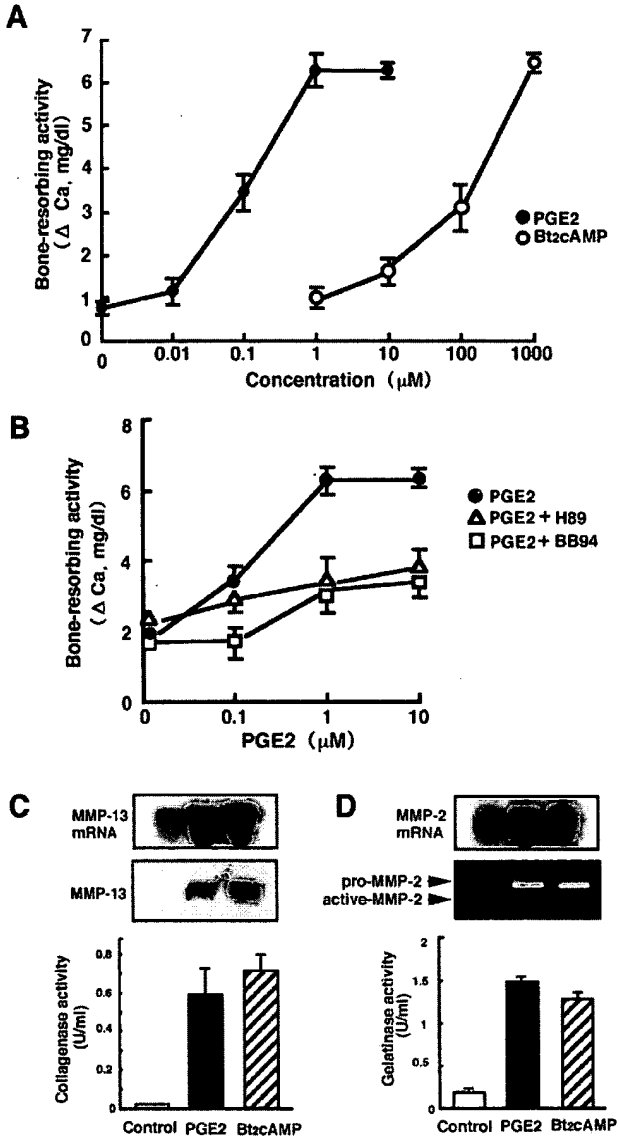

FIG. 1. **Effects of PGE_2 and Bt_2cAMP on bone resorption (*A* and *B*) and induction of MMPs in mouse calvarial cultures (*C* and *D*).** *A*, calvaria collected from 1-day-old ddy mice were cultured for 72 h with various concentrations of PGE_2 (●) or Bt_2cAMP (○). Conditioned media were collected, and calcium contents were measured. Bone-resorbing activity was expressed as the increase in the medium calcium. *B*, mouse calvariae were cultured for 72 h with PGE_2 (0.1–10 μM) in the presence or absence of 10 μM H89 (△), an inhibitor of protein kinase A, and 10 μM BB94 (□), an inhibitor of MMPs (●, PGE_2 only). Bone-resorbing activity was calculated by measuring medium calcium concentration. *C*, expression of MMP-13 mRNA and its protein in mouse calvarial cultures. Mouse calvariae were cultured for 48 h with 1 μM PGE_2 or 1000 μM Bt_2cAMP. After culture, total RNA was extracted from calvaria and Northern blotting was performed using ^{32}P-labeled cDNA probes for MMP-13 (*upper panel*). Protein lysates were extracted from calvaria and Western blotting was performed using anti-MMP-13 antibody (*middle panel*). Conditioned media were collected, and collagenase activity was measured by the degradation of FITC-labeled type I collagen after pre-treatment with 10 mM 4-aminophenylmercuric acetate to activate pro-MMPs, as described under "Materials and Methods" (*bottom panel*). *D*, expression of MMP-2 and gelatinase activity in mouse calvarial cultures. After cultures were performed under the same conditions as in *C*, the expression of MMP-2 mRNA was examined by Northern blotting (*upper panel*), and MMP-2 in the conditioned media was detected by gelatin zymography (*middle panel*) as described under "Materials and Methods." Gelatinase activity corresponding to pro-MMP-2 and active-MMP-2 is indicated by *arrows*. Conditioned media were also used to detect gelatinase activity measured by the degradation of FITC-labeled type IV collagen (*bottom panel*). Data are expressed as the means ± S.E. of four to nine independent experiments.

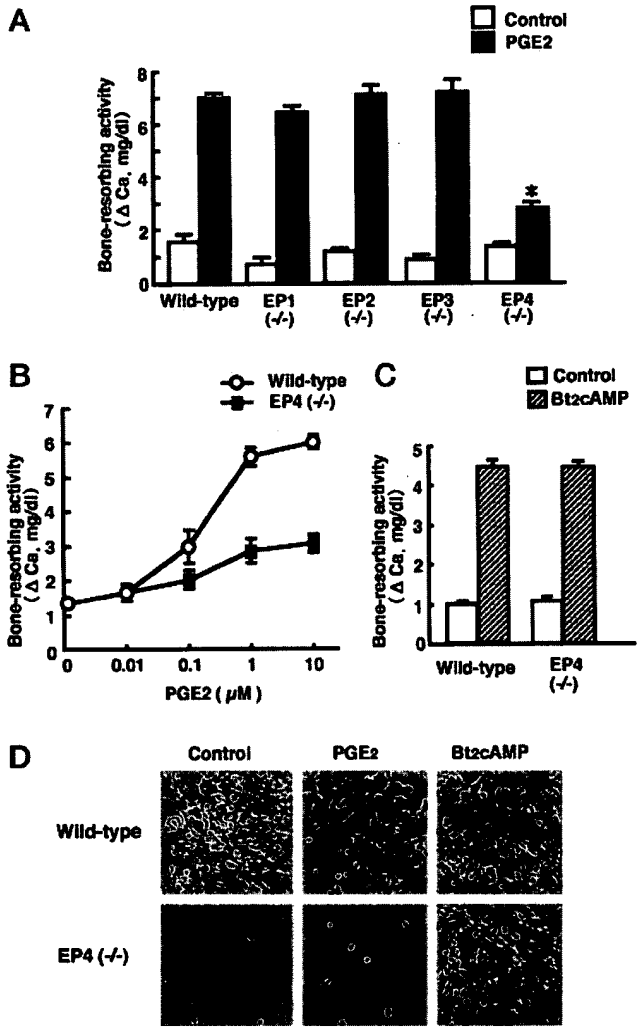

FIG. 2. **Effects of PGE_2 and Bt_2cAMP on bone resorption of calvaria from PGE receptor (*EP*) knockout mice.** *A*, mouse calvariae were collected from 1-day-old wild-type mice and from EP1 (−/−), EP2 (−/−), EP3 (−/−), and EP4 (−/−) mice, and cultured for 72 h with or without 10 μM PGE_2. Bone-resorbing activity was expressed as the increase in medium calcium as described under "Materials and Methods." Data are expressed as the means ± S.E. of 6–10 independent experiments. Results in EP4 (−/−) mice were significantly different from the cultures treated with PGE_2 in wild-type mice (*, $p < 0.001$). *B*, mouse calvaria were collected from wild-type mice (○) and EP4 (−/−) mice(■), and cultured for 72 h with 0.01–10 μM PGE_2. Bone-resorbing activity was measured. Data are expressed as the means ± S.E. of six cultures. *C*, mouse calvariae were collected from wild-type and EP4 (−/−) mice, and bone-resorbing activity induced by 1000 μM Bt_2cAMP was measured. Data are expressed as means ± S.E. of six cultures. *D*, mouse calvariae collected from wild-type and EP4 (−/−) mice were cultured for 72 h with 10 μM PGE_2 or 1000 μM Bt_2cAMP. After culture, calvariae were fixed and stained for TRAP to detect osteoclasts as described under "Materials and Methods."

with PGE_2 or Bt_2cAMP, and stained for TRAP to detect osteoclasts. In PGE_2- and Bt_2cAMP-treated calvaria, numerous TRAP-positive osteoclasts were detected in the wild-type mice (Fig. 2*D*). In contrast, in EP4(−/−) mice, osteoclasts were formed in Bt_2cAMP-treated calvaria, but not in PGE_2-treated calvaria (Fig. 2*D*). These histological findings are consistent with the bone-resorbing activities induced by PGE_2 and Bt_2cAMP shown in Fig. 2 (*B* and *C*).

Induction of bone resorption by PGE_2 was also examined in long bone cultures using EP4(−/−) and wild-type mice. PGE_2 at 0.1–10 μM dose-dependently stimulated bone resorption in long bone cultures as well. In contrast, PGE_2-induced bone

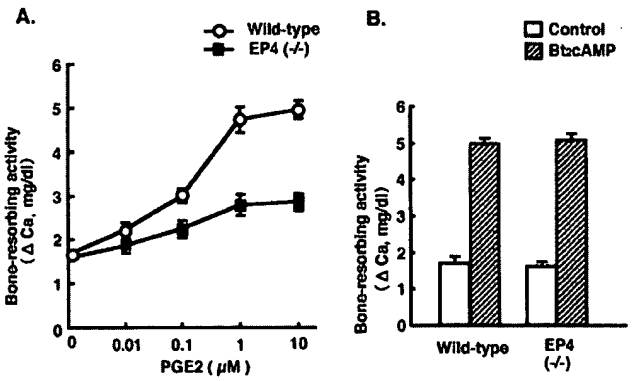

FIG. 3. **Effects of PGE_2 and Bt_2cAMP on bone resorption in mouse long bone cultures collected from wild-type and EP4 (−/−) mice.** *A*, mouse long bones were collected from 1-day-old wild-type (○) and EP4(−/−) mice(■), and cultured for 72 h with 0.01–10 μM PGE_2. *B*, mouse long bones were collected from wild-type and EP4 (−/−) mice, and cultured for 72 h with 1000 μM Bt_2cAMP. Bone-resorbing activity was expressed as the increase in medium calcium. Data are expressed as the means ± S.E. of 5–10 cultures.

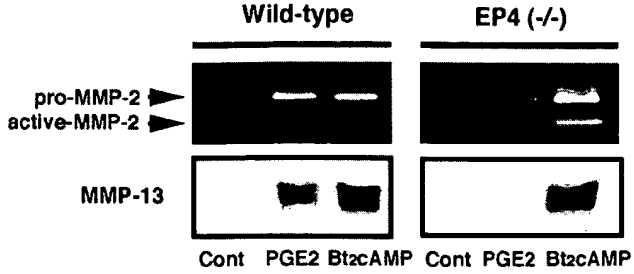

FIG. 4. **Effects of PGE_2 and Bt_2cAMP on the expression of MMP-2 and MMP-13 in mouse calvaria collected from wild-type and EP4 (−/−) mice.** Mouse calvariae were collected from 1-day-old wild-type and EP4 (−/−) mice, and cultured for 72 h with 10 μM PGE_2 or 1000 μM Bt_2cAMP. After culture, the conditioned media were collected and used for gelatin zymography to detect MMP-2 as described under "Materials and Methods" (*top panels*) and protein lysates were extracted from calvaria for Western blotting using anti-MMP-13 antibody (*bottom panels*). Gelatinase activity corresponding to pro-MMP-2 and active MMP-2 is indicated by *arrows*.

resorption was greatly impaired in long bone cultures from EP4(−/−) mice (Fig. 3*A*). Bt_2cAMP, however, greatly stimulated bone resorption both in wild-type and EP4(−/−) mice (Fig. 3*B*). These results are consistent with the data obtained by calvarial cultures shown in Fig. 2, confirming the requisite role of EP4 for PGE_2-induced bone resorption.

To further analyze the reduced bone resorption by PGE_2 in EP4 (−/−) mice, we examined the induction of MMPs by PGE_2 by Western blot analysis and gelatin zymography of the culture media of calvaria from these mice. Induction of MMP-2 and MMP-13 by PGE_2 was greatly diminished in EP4(−/−) mice compared with the wild-type mice (Fig. 4). In contrast, Bt_2cAMP similarly induced MMP-2 and MMP-13 in both EP4(−/−) and wild-type mice (Fig. 4). This indicates that the induction of MMPs is involved in PGE_2-induced bone resorption mediated by EP4.

Bone resorption is mediated by several processes, including osteoclast differentiation, fusion and activation, and MMP-dependent matrix degradation. Recently Everts *et al.* (17) reported that osteoclastic bone resorption depends on the activity of both cysteine proteinases such as cathepsin K, and MMPs in calvaria, whereas long bone resorption depends on only cysteine proteinases. This suggests that there is a difference of osteoclast function in each skeletal site. In this study, bone resorption induced by PGE_2 was diminished not only in calvarial cultures but also in long bone cultures (Figs. 2 and 3). Further studies are needed to define whether the involvement of MMPs is different between PGE_2-induced long bone resorption and calvarial bone resorption in wild-type and EP4(−/−) mice. Osteoclast formation induced by PGE_2 was diminished in EP4(−/−) mice both in calvarial cultures (Fig. 2*D*) and in bone marrow cultures (data not shown). Therefore, the process of osteoclast differentiation stimulated by PGE_2 may also be involved in the mechanism of impaired bone resorption to PGE_2. More recently, Sakuma *et al.* (18) reported that osteoclast formation was diminished in the coculture of osteoblastic cells from EP4(−/−) mice and spleen cells from wild-type mice. These findings indicate that PGE_2 stimulates bone resorption by a cAMP-dependent mechanism mediated by EP4, and that the induction of MMPs and osteoclast formation are involved in bone resorption induced by PGE_2.

As reported previously, all EP1(−/−), EP2(−/−), EP3(−/−), and EP4(−/−) mice are born at the predicted Mendelian frequency. EP1(−/−), EP2(−/−), and EP3(−/−) mice grow normally, and no apparent defects or abnormality is detected in bone by the soft x-ray analysis (data not shown). Most EP4(−/−) neonates die within 72 h after birth by patent ductus arteriosus (11), which has precluded an examination of bone tissues in adult EP4(−/−) mice. Reverse transcriptase-polymerase chain reaction analysis indicated that osteoblast-like cells isolated from calvaria of wild-type newborn mice expressed all EPs mRNA, and the order of the expression levels was EP4 > EP1 > EP2 > EP3 (data not shown). Because EP2 and EP4 stimulate adenylate cyclase in several types of cells, and cAMP production by osteoblasts is thought essential for the induction of bone resorption by PGE_2 (5–8), EP2 and EP4 have been considered as the most likely receptors to mediate bone-resorbing activity of PGE_2. In this study, we have found a marked reduction of bone-resorbing activity by PGE_2 only in the bone from EP4(−/−) mice. These observations indicate that PGE_2 stimulates bone resorption mainly by a cAMP-dependent mechanism involving EP4. It should be noted, however, that the PGE_2-induced bone-resorbing activity was not completely abolished in EP4 (−/−) mice. Some activity induced by PGE_2 remained in EP4(−/−) mice. Thus, a possible involvement of other EPs in PGE_2-induced bone resorption cannot be excluded at present.

PGE_2 is known to be a critical factor in bone formation and resorption *in vivo* and *in vitro* (2, 3, 5, 19). Recent studies suggest that PGE_2 is involved in the pathogenesis of certain metabolic bone diseases including osteoporosis (20, 21). Cytokines such as IL-1 and IL-6 have bone-resorbing activities and are likely involved in the pathogenesis of osteoporosis (20, 22–25). Their bone-resorbing actions are at least partly dependent on PGE_2 production induced by these cytokines in osteoblasts. One way to control PGE_2-dependent bone resorption may be therefore to regulate PGE_2 production by osteoblasts. It is known that PGE_2 synthesis is regulated by the cytosolic phospholipase A_2-dependent release of arachidonic acid and the COX-2-catalyzed conversion of arachidonic acid into PGH_2 (4, 26–28). COX-2 inhibitors have been therefore regarded potential candidates for the treatment of PGE_2-dependent bone resorption. This study suggests an alternative possibility that specific antagonists for EP4 may be useful in regulating PGE-mediated metabolic bone diseases. This possibility is currently being explored in our laboratories.

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